Patent
Attorney's Docket No. 032425-001

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

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In re Patent Application of )
Marshall S. Horwitz et al )
)
Application No.: 09/132,231 ) Group Art Unit: 1632
)
Filed: August 11, 1998 ) Examiner: J. Brusca
)
For: METHOD FOR PRODUCING )
NOVEL DNA SEQUENCE WITH )
BIOLOGICAL ACTIVITY )

## DECLARATION PURSUANT TO 37 C.F.R. § 1.608

I, Phillip A. Patten, solemnly swear and attest to the truth of the following:

(1) I am employed as a Scientist at Maxygen Inc., and have been employed in this position for 4 years.

(2) I am a person of skill in the art of molecular biology, and particularly technology concerning evolutionary biotechnology, and have been quite familiar with this art for at least 18 years as evidenced by the attached curriculum vitae (references that are most relevant to applied molecular evolution technology or antibody affinity maturation are indicated in bold).

(3) I have read the Pieczenik patent (US 5,866,363), and am quite familiar with the technology discussed in it. I am also familiar with the technology discussed in the Horwitz patent application 09/132,231.

(4) I have been asked to provide my comments on what aspects of the Pieczenik disclosure are required for the identification of short random peptides (4-12 amino acids) by random antibodies. In particular, I have been asked to discuss whether the methods in Pieczenik could

be performed without the filamentous phage vector system used in Example 4 of the patent. In this regard, it has been explained to me that this Example was not present in the original Pieczenik application filed August 28, 1985, and that Pieczenik added this Example in a later application. I have reviewed the Pieczenik patent to determine whether a person skilled in the art at the time of the original application could have practiced the subject matter claimed in the Pieczenik patent without having the information provided in Example 4.

(5) As a result of my review, I believe that the filamentous phage vector system used in Pieczenik, or a similar expression system, would have been required to screen a randomly-generated epitope library with random antibodies. Pieczenik first proposes using a lambda gt11 expression system. Although similar expression systems are commonly used to screen cloned DNA for genes expressing *particular* peptides or proteins using high affinity antibodies as probes, I fail to see how such a system could be employed for Pieczenik's methods. As detailed below, it would have been extremely difficult, if not impossible, to identify particular antibodies from a non-immunized animal that identify particular peptides within a randomly-generated population using a lytic phage expression system and the screening methods available in 1985.

(6) In the Detailed Description of the Invention (column 6, lines 65 and following), Pieczenik asserts that "According to the clonal selection theory, an unchallenged mammalian host has the capacity to produce antibodies to a vast array of foreign antigens. The presence of an antigen triggers the proliferation of those lymphocytes already present having the ability to produce antibodies specific for that antigen. ..... Thus it can be expected that each mammal has the capability to produce antibodies that will recognize most, if not all of these sequences. Thus, the spleen of a mouse or another laboratory animal can serve as an appropriate source of a full range of antibodies." This assertion is incorrect in that it does not take into account the fundamental phenomenon of affinity maturation that occurs during immunization with a specific antigen (or during an infection).

(7) Affinity maturation is a natural process of hypermutation and selection that occurs in vivo during immunization and typically leads to an increase of one to several orders of magnitude in binding affinity for the immunizing antigen [Siskind, G.W. and Benaceraff, Adv. Immunol. 10:1 (1969), Kim et. al., Cell 27:573 (1981), Tonegawa, S. Nature 302:575 (1983)]. In a recent study, affinity maturation of an antibody was reconstructed in detail by comparing the biochemical properties of a high affinity antibody that was the product of immunization to that of the "germline" antibody that was reconstructed from the unmutated V-D-J gene segments from a Balb/c mouse (Patten, P.A. et al Science 271:1086 (1996)). In this case, the affinity matured antibody had accumulated nine mutations during affinity maturation, and the affinity improved by 30,000-fold as a result of this maturation process (from 130 micromolar to 5 nanomolar). The antisera from unimmunized mice described by Pieczenik would be expected to have low affinity for any antigens that they bind, as is characteristic of antibodies from unimmunized mice, making library screening very challenging.

(8) Selecting random monoclonal antibodies that bind specifically to recombinant epitopes expressed in lytic expression systems would be impractical by the methods described by Piecznik in 1985. Antibodies from unimmunized mammalian immune systems are often "polyreactive," binding to multiple antigens (J. Immunol. 135:3122-7. 1985). This polyreactive property of antibodies from naïve repertoires would confound the screening of libraries of random epitopes because polyreactivity would lead to a large amount of "background" binding to E. coli proteins.

(9) For instance, phage lambda gt11 lyses the host cell as it multiplies, releasing all the cell's contents (including other proteins). It might be feasible to identify specific clones that encode specific peptides corresponding to a protein used for immunization using the antisera from an animal immunized with a specific antigen, because such specific antibodies would be present at an increased frequency in the antisera, and would have undergone somatic mutation and affinity maturation. However, if the antibodies being screened were truly "random," i.e., none are present at an increased frequency and none have undergone affinity maturation, then antibodies that recognize any given bacterial peptide or protein will be just as prevalent as antibodies that recognize the encoded "random" peptide. It would take a significant amount of screening each

antibody identified against cells lysed with phage alone (no encoded peptide), and then without phage, to identify an antibody that actually recognizes the peptide encoded by the random piece of DNA inserted into the phage vector.

(10) As a first-order approximation, E. coli has a genome of $3x10^6$ base pairs, of which about $1x10^6$ base pairs encode gene products expressed during lytic infection. This expressed sequence encodes about $5x10^4$ 5-10 amino acid peptides. It is important to also realize that a random population of antibodies, i.e., formed as a result of in vitro expansion of cloned repertoires of antibodies, rather than immunization, would also include antibodies that happen to recognize phage lambda proteins. Thus, one could not simply verify that any given antibody recognized a random peptide encoded by a cloned DNA rather than a phage protein without comparing the binding of the antibody to cellular proteins released by means of infection of the lambda vector carrying no cloned insert. These expressed peptides will give rise to "background" against which "signal" must be detected in a reaction of an antibody with a peptide immobilized matrix (an "A-PIM", in the parlance of Pieczenik). Thus, even if one could identify an antibody that recognizes a plaque produced by a lytic phage vector encoding a random epitope, one would have much difficulty identifying interactions that are specific to cloned epitopes.

(11) In the 1985 invention, the authors describe two types of antibody probes: polyclonal antisera and collections of hybridomas derived from either naïve or immunized mice. Neither format enables practical methods for obtaining antibodies that bind specifically and with high affinity to cloned epitopes. The frequency of B cells that bind to an antigen with which the animal has not been immunized is about $10^{-5}$ to $10^{-6}$ (based on the measured frequency of antigen specific B cells in spleens). These low frequencies alone make it impractical to screen random hybridomas for binders to particular antigens of interest (I am not aware of any research team in academia or industry screening $10^5$ hybridomas for binders to an antigen of interest. In fact, 1000 is considered in the field to be a large screen). In addition to this practical limitation and as detailed above, these antibodies to antigens to which the animal has not been exposed will not have been subjected to affinity maturation, and they will therefore typically be of low affinity and often be polyreactive.

(12) It is only with the advent of very high complexity ($<10^9$) display libraries and powerful biopanning protocols such as phage display that it has been possible to obtain antibodies with moderate affinity (better than 100 nM) from naïve combinatorial libraries. Use of a filamentous phage vector system, however, enables the expression of peptides on the cell surface by virtue of fusions to phage coat proteins. Thus, no lysis of the cell is required to screen antibodies, and there is no confusion with cellular proteins and peptides. In this regard, however, it is not clear to me why one would want to identify antibodies that bind to random peptide sequences. I understand that the goal of Horwitz was to identify new proteins and peptides from a random population that happen to have certain functions in the absence of evolution. Pieczenik seeks to categorize random antibodies according to their binding specificity (see the Background section of the patent and the discussion as to how "the spleen can be considered a library of cells"). But such antibodies have no valid use unless one can identify a "real" peptide or protein (as an actual marker for disease, for instance) for which the identified antibody may be used for instance in a diagnostic or experimental capacity.

(13) Furthermore, it seems the only way to do this would be to either screen the antibodies with a known protein in the first place, or keep a bank of antibodies that recognize certain specific peptide sequences, whereby the bank can be consulted for antibodies which bind to newly discovered proteins by virtue of sequence identity between the proteins and identified peptides. This use would depend on a strong correlation between binding to a linear peptide and binding to a peptide of the same sequence which is embedded within a folded protein. There is a great deal of literature to suggest, however, that antibodies raised against linear peptides generally do not react strongly with native proteins containing these linear epitopes.

(14) For example, the abstract of a paper (Z. Naturforsch 40:148-9, 1985) by the prominent peptide immunochemist R. Houghten reads as follows: "Rabbit antibodies against small peptides may be composed by subpopulations recognizing different epitopes made likely by few amino acids. This explains the frequent crossreactivity of antipeptide antibodies with unrelated peptides. A suitable use of immunoadsorbents is suggested to obtain truly specific antibodies able to react with restricted amino acid sequences." Thus, even if one is able to

identify antibodies that recognize linear peptide sequences, there is no guarantee that such antibodies will recognize or have the same affinity for the same linear sequence embedded within a folded protein.

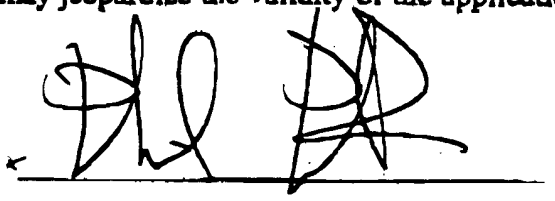
(15) Pieczenik also mentions using polyclonal antibody sera to bind antigens of interest and then eluting these antibodies off of the antigen. I fail to see how one would work backward from such populations of antibodies to clone or isolate monoclonal populations of antibodies against the epitope of interest.

(16) In summary, without a consideration of the methods and concepts described above, which were not described in the original Pieczenik application (1985) or the later filed application for that matter, it would not be practical to obtain, from naïve antibody libraries, antibodies against specific antigens of interest, let alone multiple epitopes or multiple proteins of interest. While one might have a better chance of identifying antibodies which recognize random peptides using a filamentous phage vector system versus a lytic phage system, the expected low frequency of antigen binding to antibodies in naïve libraries and low affinity and polyreactivity of these antibodies to random peptide would make the screening of libraries of random peptides impractical even in this context.

(17) I further declare that all statements made herein of my knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application and any patent issuing therefrom.

1-7-2000

Date



Signature

# PHILLIP ANDREW PATTEN

Director

Protein Pharmaceuticals Maxygen Inc.

Mailing address:
261 La Cuesta Drive
Menlo Park, CA 94028

Telephone: (408) 522-6079
e-mail: phil_patten@maxygen.com

**Date of Birth:** May 31, 1960

**Place of Birth:** Seattle, Washington

**Citizenship:** U.S.A.

**Social Security Number:** 533-74-1881

**Educational Background:**

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|---|---|
| 9/78 - 8/79 | University of Washington |
| 9/79 - 6/82 | California Institute of Technology<br>Bachelor of Science with Honors, Biology |
| 9/83 - 6/90 | Stanford University<br>Department of Biological Sciences, Ph.D. |

**Professional Experience:**

6/80 - 8/80 Undergraduate Res. Assistant, Molecular Immunology,

Dr. Leroy Hood Laboratory, California Insiiute of Technology

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|---|---|
| 6/81 - 8/81 | Research Assistant, Primate Endocrinology,<br>Dr. Charles Gale Laboratory, University of Washington |
| 9/81 - 6/82 | Undergraduate Research Assistant, Elliot Meyerowitz Laboratory, California Institute of Technology |

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|---|---|
| 9/82 - 8/83 | Research Scientist, plant genetic engineering Phytogen, Inc., Pasadena, California |
| 9/83 - 6/90 | Ph.D. Candidate, Stanford University |
| 7/90 - 3/96 | Damon Runyon Postdoctoral Fellow, Department of Chemistry, U.C. Berkeley |
| 4/96 - 3/97 | Visiting Scientist, Affymax Research Institute |
| 4/97 - 12/97 | Staff Scientist I, Maxygen, Inc. |
| 1/98 – 10/98 | Staff Scientist II, Maxygen, Inc. |
| 10/98 – 10/99 | Senior Scientist and Group Leader, Protein Pharmaceuticals, Maxygen Inc. |
| 10/99 – present | Director, Protein Pharmaceuticals |

**Academic Awards and Honors:**

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| 1978 | National Merit Scholar |
| 1981 | Scholarship, Achievement Rewards for College Scientists |
| 1981 | Undergraduate Research Award, Swedish Club of Los Angeles |
| 1982 | Bachelor of Science with Honors, California Institute of Technology |
| 1989 | Damon Runyon - Walter Winchell Cancer Research Postdoctoral Fellowship, Sponsor: Jeremy Knowles, Harvard University (forfeited) |
| 1990 | American Cancer Society Postdoctoral Fellowship Sponsor: Dr. Peter Schultz, U.C. Berkeley (forfeited) |
| 1990 | Damon Runyon - Walter Winchell Cancer Research Postdoctoral Fellowship, Sponsor: Dr. Peter Schultz, U.C. Berkeley |

**Publications:**

1. **Kim, S., Davis, M., Sinn, E., Patten, P., and Hood, L. Antibody Diversity: Somatic Hypermutation of Rearranged V-H Genes.** *Cell* **27:573-581 (1981).**

2. Thomas, J.B., Patten, P.A., and Goodman, C.S. Neuronal development in the Drosophila embryo: Subtracted cDNA as a probe for potential recognition molecules. *Soc. Neuro. Abstr.* 10:141 (1984).

3. Patten, P., Yokota, T., Rothbard, J., Chien, Y., Arai, K., and Davis, M. Structure, expression and divergence of T-cell receptor β-chain variable regions. *Nature* 312:40-46 (1984).

4. Becker, D., Patten, P., Chien, Y., Yokota, T., Eshhar, Z., Giedlin, M., Gascoigne, N., Goodnow, C., Wolf, R., Arai, K., and Davis, M. Variability and repertoire size of T-cell receptor V-α gene segments. *Nature* 317:430-434 (1985).

5. Gascoigne, N., Chien, Y., Patten, P., Becker, D., Lindsten, T., Kavaler, J., Lee, N., and Davis, M. The genes of the murine T cell receptor. In *Human T Cell Clones*, Feldman, M., Lamb, J.R., and Woody, J.N. (eds.), Humana Press, Clifton, New Jersey, 25-34 (1985).

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7. Elliot, J.E., Rock, E.P., Patten, P.A., Davis, M.M., and Chien, Y. The adult T- cell receptor δ-chain is diverse and distinct from that of fetal thymocytes. *Nature* 331:627-631 (1988).

8. Davis, M.M., Chien, Y-H., Bjorkman, P.J., Elliott, J.F., Iwashima, M., Rock, E.P., and Patten, P.A. A Possible Basis for Major Histocompatibility Complex- Restricted T-Cell Recognition. *Philos. Trans. R. Soc. London. B Biol. Sci.* 323 (1217): 521-524 (1989).

9. Patten, P.A., Doctoral Dissertation: Descriptive Studies and Genetic Analysis of the Murine αβ T Cell Receptor *and* An Experimental Approach to Molecular Evolution, Stanford University, Department of Biological Sciences (1990).

10. Patten, P.A., Rock, E.P., Sonoda, T.S., Fazekas de St. Groth, B., Jorgenson, J., and Davis, M.M. Transfer of Putative CDR Loops of T Cell Receptor V Domains Confers Toxin Reactivity, but not Peptide Specificity. *J. Immunology* 150:2281- 2294 (1993).

11. Fazekas de St. Groth, B., Patten, P.A., Ho, W.Y., Rock,E.P., and Davis, M.M. An Analysis of T Cell Receptor-Ligand Interaction Using a Transgenic Antigen Model for T Cell Tolerance and T Cell Receptor Mutagenesis in *Molecular Mechanisms of Immunological Self-Recognition*, Academic Press, Inc. (1993).

12. Lesley, S.A., Patten, P.A., and Schultz, P.G. A genetic approach to the generation of antibodies with enhanced catalytic activities. *Proc. Natl. Acad. Sci.* 90: 1160-1165 (1993).

13. Ullrich, H. D., Patten, P. A., Yang, P. L., Romesberg, F. E., and Schultz, P. G., Expression studies of catalytic antibodies. *Proc. Natl. Acad. Sci.* 92: 11907-11911 (1995).

14. Patten, P. A., Sonoda, T. and Davis, M. M. Directed evolution studies with combinatorial libraries of T4 lysozyme mutants. *Molecular Diversity* 1:97-108 (1995).

15. Patten, P.A., Gray, N., Yang, P. L., Marks, C. B., Wedemayer, G. J., Boniface, J. J., Stevens, R. C., and Schultz, P. G. The Immunological Evolution of Catalysis. *Science* 271:1086-1091 (1996).

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Wedemayer, G. J., Patten, P. A., Wang, L. H., Schultz, P. G., and Stevens, R. C. Structural Insights into the Evolution of an Antibody Combining Site. *Science* 276:1665-1669 (1997).

Patten, P.A., Howard, R.J., and Stemmer, W.P.C. Applications of DNA Shuffling to Pharmaceuticals and Vaccines. *Current Opinion in Biotechnology* 8 (6):724-733 (1997).

Chang, C.J., Chen, T.T., Cox, B.W., Dawes, G.N., Punnonen, J. and Patten, P.A. Rapid Evolution of a Cytokine using Molecular Breeding. *Science* (submitted).

## Grants

Principle investigator on $2 million grant from the Advanced Technology Program (General Competition)

Evolution of a murine model for AIDS: Applications to discovery of small molecule and vaccine therapeutics.

### Pending Patents

Six patents pending on the application of DNA shuffling to various areas of biotechnology.